The Mitochondrial Genome of *Phoronis architecta*—Comparisons demonstrate that Phoronids are Lophotrochozoan Protostomes

Kevin G. Helfenbein[1] and Jeffrey L. Boore

Department of Biology, University of Michigan, 830 N. University Ave., Ann Arbor, MI

48109 USA

and

DOE Joint Genome Institute and Lawrence Berkeley National Laboratory, 2800 Mitchell

Drive, Walnut Creek, CA 94598 USA

[1]Present address: American Museum of Natural History, Department of Invertebrate

Zoology

Corresponding author: Jeffrey L. Boore, DOE Joint Genome Institute, 2800 Mitchell

Drive, Walnut Creek, CA 94598, telephone:925-296-5691, Fax: 925-296-5666,

JLBoore@lbl.gov

Abstract

The proper reconstruction of the relationships among the animal phyla is central to interpreting patterns of animal evolution from the genomic level to the morphological level. This is true not only of the more speciose phyla, but also of smaller groups. We report here the nearly complete DNA sequence of the mitochondrial genome of the phoronid *Phoronis architecta*, which has a gene arrangement remarkably similar to that of a protostome animal, the chiton *Katharina tunicata*. Evolutionary analysis of both gene arrangements and inferred amino acid sequences of these taxa, along with those of three brachiopods and other diverse animals, strongly supports the hypothesis that lophophorates are part of the large group that includes mollusks and annelids, i.e. the Lophotrochozoa, and solidly refutes the alternative of their being deuterostomes.

Introduction

The group Lophophorata contains three animal phyla—brachiopods, phoronids and ectoprocts—all of which bear a ciliated, tentacular feeding apparatus called a lophophore (Hyman 1959). The phylogenetic position of the lophophorates has long been controversial. Various studies have placed these taxa in widely disparate branches of the metazoan tree—in morphological analyses among the deuterostome animals (e.g. chordates and echinoderms) (Nielsen, Scharff, and Eibye-Jacobsen 1996; Brusca and Brusca 2003), but in ribosomal RNA sequence analyses among the protostomes (e.g. annelids and mollusks) (Field et al. 1988; Halanych et al. 1995; Mackey et al. 1996; Cohen, Gawthrop, and Cavalier-Smith 1998; Cohen et al. 1998). Most researchers have come to favor the latter view, although there remains controversy, including finding a deuterostome affinity for the lophophorates in a very recent, comprehensive morphological analysis (Brusca and Brusca 2003). Among the phylogenetic studies that support this position, the strongest evidence has come an analysis of the inferred amino acids of six mitochondrially encoded genes from metazoan taxa including the articulate brachiopod *Terebratulina retusa* (Stechmann and Schlegel 1999).

The number of complete mitochondrial DNA (mtDNA) sequences is growing rapidly. Among the triploblast animals, this circular molecule ranges in size from about 13 to 18 kb and almost always contains the same 37 genes: 13 for proteins, 22 for tRNAs, and two for rRNAs (Boore 1999). For some groups of animals, the arrangement of these genes is very stable over long periods of time; for example, all 37 genes of human and shark mtDNA are identically arranged (Boore 1999). For other lineages, rearrangements are much more rapid; for example, other than the short block *trnL1-trnL2-nad1*, there are

no gene boundaries in common between the mitochondrial genomes of two mollusks, a chiton (class Polyplacophora) *Katharina tunicata* (Boore and Brown 1994) and a mussel (class Bivalvia) *Mytilus edulis* (Hoffman, Boore, and Brown 1992).

In recent years, the comparison of complete mitochondrial genomes has become a very powerful tool for reconstructing phylogeny. The comparisons of complete mitochondrial genome sequences have been shown to have much greater resolving power than for individual gene sequences (Cao et al. 2000; Ingman et al. 2001; Miya, Kawaguchi, and Nishida 2001). In addition, the relative arrangement of these 37 genes can be an especially powerful tool for phylogenetic analysis (Boore et al. 1995; Boore and Brown 1998, 2000). The great number of potential gene rearrangements makes it very unlikely that different animal lineages would independently adopt the same gene order or that any gene would move back to a previous location, so gene arrangements that are shared in a derived condition (see below) are a very strong indicator of relatedness (Boore and Brown 1998).

Among lophophorate taxa, the only complete mitochondrial genome sequences available are for three brachiopods, *Terebratulina retusa* (Stechmann and Schlegel 1999), *Laqueus rubellus* (Noguchi et al.. 2000), and *Terebratalia transversa* (Helfenbein, Brown, and Boore 2001). Although similarity of gene arrangement to non-lophophorate taxa was noted in these studies, no formal phylogenetic analysis was performed. Phoronida is a second lophophorate phylum, comprised of coelomate, vermiform animals living in chitinous tubes. Here we report the sequence of nearly all (i.e. 14,018 nts) of the mitochondrial genome of the phoronid *Phoronis architecta*, from which we can

determine the arrangements of all 13 of the expected protein encoding genes, both of the rRNA genes, and 16 of the expected 22 tRNA genes.

Materials and Methods

DNA isolation, PCR amplifications, and DNA sequencing

Specimens of *Phoronis architecta* were purchased from Gulf Specimen laboratories, Inc. Total genomic DNA was isolated from a single individual by standard techniques. Regions within *cob* and *rrnS* and spanning *cox1* to *cox2* were amplified with primers designed to conserved sequences (Boore and Brown 2000). The sequences from these amplified regions were used to design specific primers for long PCR. Part of the genome was amplified by long PCR (*rrnS* to *cob*), and part by a combination of step-out PCR (using a combination of one random and one specific primer), short PCR (*cox1*, *cox3*, and *nad4)* and long PCR (*cox1-nad4*, *cox1-cox3*, and *nad4-cob).* Sequences were obtained by primer walking through the long PCR amplifications using an ABI 377 automated DNA sequencer. All chromatograms were examined by eye to verify sequencing accuracy. Multiple attempts were made to amplify the region *rrnS-cox3* using a variety of reaction conditions, but all failed. It is possible that this region contains sequences that are recalcitrant to amplification or that it is larger than expected.

Sequence analysis

Sequences were produced and assembled using the ABI suite of programs (e.g., Sequencing Analysis™, Sequence Navigator™, Autoassembler™). Subsequent manipulations used MacVector™ 6.5 (Oxford Molecular Group). Protein encoding genes

were identified by BLAST matching to other animal mtDNAs, with start codons inferred as being an eligible in frame start codon corresponding at least to the extent of alignment that does not overlap the upstream gene. Abbreviated stop codons, presumably completed by polyadenylation of the mRNA after cleavage, were inferred in cases where extensive gene overlap beyond the extent of homologous gene sequence matching would be required to reach the first complete stop codon. Ribosomal RNAs were identified by BLAST matching. Transfer RNA genes were identified generically by their potential secondary structures and specifically by anticodon sequence, in some cases using the tRNAscan-SE Search Server (www.genetics.wustl.edu/eddy/tRNAscan-SE).

Sequences and alignments

We chose a broad representation of taxa from available complete mtDNA sequences for this study. All of the mtDNAs of the triploblast taxa contain unambiguous copies of the 37 genes mentioned above. The following sequences were retrieved from GenBank: *Terebratulina retusa* (accession number NC_000941), *Laqueus rubellus* (NC_002322), *Terebratalia transversa* (NC_003086), *Platynereis dumerilii* (NC_000931), *Lumbricus terrestris* (NC_001673), *Loligo bleekeri* (NC_002507), *Katharina tunicata* (NC_001636), *Drosophila yakuba* (NC_001322), *Daphnia pulex* (NC_000844), *Locusta migratoria* (NC_001712), *Limulus polyphemus* (NC_003057), *Paracentrotus lividus* (NC_001572), *Asterina pectinifera* (NC_001627), *Florometra serratissima* (NC_001878), *Balanoglossus carnosus* (NC_001887), *Homo sapiens* (NC_001807), *Mustelus manazo* (NC_000890), *Metridium senile* (NC_000933), *Podospora anserina* (NC_001329), and *Pichia canadensis* (NC_001762).

Inferred amino acid sequences of nine mitochondrial protein genes (*atp6*, *cob*, *cox1*, *cox2*, *cox3*, *nad1*, *nad3*, *nad4*, and *nad5)* from 18 ingroup and three outgroup taxa were aligned using ClustalW. Pairwise alignments were done in slow mode with an open gap penalty of 10 and an extend gap penalty of 1 using the BLOSUM similarity matrix. The multiple alignment used the same parameters as the pairwise plus a "delay divergent sequences" setting of 40%. Ambiguously aligned positions at the termini of each gene alignment were trimmed. Gaps were included in the analysis and scored as "missing characters". (See supplementary material online).

Phylogenetic reconstruction

Parsimony analysis of aligned inferred amino acid sequences employed a heuristic search with 10,000 random additions in PAUP* version 4.01b10 (Swofford 1998). Bootstrap values were calculated using 500 replicates with an addition sequence of 10 random replicates. Bremer branch support values (i.e. decay indices; Bremer 1994) were calculated using Tree Rot (Sorenson 1996). Maximum-likelihood analysis of the same alignment employed 10,000 quartet puzzling steps, an mtREV24 model of substitution, and 8 Gamma rate categories in Tree-Puzzle version 5.0 (Strimmer and von Haeseler 1996).

For phylogenetic analysis of gene arrangements, a gene adjacency matrix was constructed for 18 animal mtDNAs as in Boore et al. (1995) in their analysis of arthropod gene arrangements. Briefly, this scores 74 characters as being "upstream of" and "downstream of" (according to transcriptional orientation) each of the 37 genes. For each character there are 72 possible character states, each being the 5' or 3' end of one of the

other 36 genes. This matrix is then analyzed using parsimony criteria with PAUP*
version 4.01b10 (Swofford 1998). This is essentially identical to the technique termed
"maximum parsimony of multiple encodings" (MPME) by Wang et al. (2002).
Boundaries not identified for the Phoronis mtDNA were scored as missing data.

The bootstrap consensus tree of the gene adjacency matrix was calculated using
1000 replicates with an addition sequence of 10 random replicates. No outgroups were
used in this analysis since no sequenced mtDNA from an appropriate taxon has the same
gene content as the triploblast animals studied here. All of the gene arrangements used in
this study can be found listed in the Mitochondrial Gene Arrangement Source Guide
(follow the Evolutionary Genomics link from http://www.jgi.doe.gov/).

Results and Discussion

We produced the sequence of 14,018 contiguous nts of the mtDNA of the phoronid
Phoronis architecta (GenBank accession number AY368231). This portion of the
mtDNA contains all or part of the two rRNA genes, the 13 protein encoding genes, and
16 of the 22 tRNA genes typically found in animal mtDNA. Despite many attempts, we
were unable to amplify the portion of the genome spanning *rrnS-cox3*, which presumably
contains the six unaccounted for tRNAs (assuming the gene content conforms to that of
most other mtDNAs; Boore 1999), those being *trnM*, *trnC*, *trnY*, *trnW*, *trnQ*, and *trnG*,
along with perhaps a large non-coding region often referred to as the "control region",
since it is thought to contain regulatory signals.

Remarkably, the arrangement of the 31 genes in the *Phoronis architecta* mtDNA is
nearly identical to that of the mollusk *Katharina tunicata* (Boore and Brown 1994). Only

three of these 31 genes are arranged differently between these two mitochondrial genomes (fig. 1). The six unaccounted for tRNA genes of *P. architecta* are clustered in the *K. tunicata* mtDNA and are between *rrnS* and *cox3*, inviting the speculation that they may also have similar or identical arrangement in the phoronid mtDNA.

There are two alternative interpretations of this surprising similarity in gene arrangement between a phoronid and a mollusk. It may be that this is a robust indicator of relatively close relationship, i.e. these phyla may share a nearly identical gene arrangement because they are more closely related to each other than either is to the animals that differ, such that they inherited this condition uniquely. Alternatively, this shared arrangement could be retained from an ancient ancestor that is common to a larger group, such that the gene arrangements of other studied animals have simply changed. These alternatives can be differentiated through a cladistic analysis, which determines whether features are "shared and derived" (synapomorphies) vs. "shared and ancestral" (sympleisiomorphies). An analysis of mitochondrial gene arrangements testing brachiopod relationships has been previously performed (Endo 2001); however, this analysis used a phenetic approach rather than a cladistic approach, which does not make this important distinction. Also, because it did not include any tRNA data, it was limited as a measurement of distance among metazoan mitochondrial gene arrangements.

Our cladistic analysis of the gene adjacency matrix for 18 animals resulted in 108 trees of equal length. The strict consensus of the 108 trees includes the lophophorates among the protostomes in a lophotrochozoan clade with 98% bootstrap support (fig. 2). This data set includes 73 parsimony informative characters, i.e. gene junctions that are shared by members of one clade, but for which other taxa share an identically rearranged

state. For example, preceding *trnK* of *Katharina* and *Phoronis* is *cox3*, whereas preceding *trnK* of *Limulus*, many vertebrates, acorn worm, and echinoderms is *cox2*. For another example, *Katharina*, *Phoronis*, and *Limulus* all share the gene arrangement *trnR-trnN*, whereas vertebrates, acorn worm, and echinoderms share the arrangement *trnR-nad4L*.

Phoronis and *Katharina* share a DNA inversion relative to the arrangement of another protostome, the arthropod *Limulus* (a horseshoe crab) (fig. 1). By assuming the same root on the gene arrangement tree as on the amino acid tree (fig. 3), and by noting that the gene junction *trnT-trnP* is present at the inversion junction in *Limulus* and is also present in the outgroup taxa Vertebrata and Enteropneusta, we have polarized this inversion and infer that the Lophotrochozoa arrangement is derived. Notably, no gene boundary characters support *Phoronis* as a deuterostome. Using MacClade (Maddison and Maddison 2000) we determined that positioning *Phoronis* as part of the deuterostome clade would require 11 additional evolutionary steps over what is required for the consensus topology (fig. 2).

We also performed parsimony and maximum-likelihood analyses using the aligned amino acids of nine of the 13 mitochondrially encoded proteins, excluding the alignments of four proteins that we subjectively determined to be too ambiguous. The topology of the most parsimonious tree (fig. 3) shows three major branches of triploblast animals as has been found in other analyses (e.g. Halanych et al. 1995; Mackey et al. 1996; Aguinaldo et al. 1997), one of which, the Lophotrochozoa, include brachiopods, phoronids, and animals that develop from a trochophore larva. The node uniting the lophophorate animals with other protostomes is well supported, with the shortest tree

lacking this node being 89 steps longer than that shown in figure 3. The maximum-likelihood analysis also strongly supports this view.

Although there is strong evidence in the mtDNA data presented here for a protostome clade containing phoronids, brachiopods, annelids, and mollusks, there is not enough phylogenetic information in the data set to resolve the relationships within the group. Of particular interest is the question of lophophorate monophyly. Halanych and co-workers found evidence of a clade of phoronids and articulate brachiopods (Halanych et al. 1995), but this result was severely questioned (Conway Morris et al. 1996). Some evidence for monophyly of brachiopods + phoronids has been presented (e.g. Cohen 2000), but more analyses of nuclear genes are needed to resolve this question and other questions of relationships within the Lophotrochozoa.

From the results of these analyses, it appears that the morphological and embryological features thought to indicate a close lophophorate-deuterostome relationship such as the mouth deriving from a secondary body opening rather than from the blastopore, the presence of ciliary bands formed by monociliate cells, the position of the nerve concentration/brain, the presence of a tripartite coelom, and other features (Nielsen, Scharff, and Eibye-Jacobsen 1996; Brusca and Brusca 2003) have either arisen independently or have been lost multiple times within the triploblastic animals. Indeed, in so strongly corroborating the phylogenetic position of phoronids and brachiopods as protostomes with this study, we would agree that morphological traits (e.g. egg cleavage patterns, modes of coelom formation, mouth formation, and trimery) are either unreliable characters for deep metazoan phylogeny, too open to interpretation as to their character states, or not polarizable, as has been suggested previously (De Rosza 2001). That the

morphological characters commonly used for metazoan systematics have not evolved as conservatively as had been believed is also suggested by a recent study of the Myzostomida (Eeckhaut et al. 2000).

Resolution of the relationships within the Lophotrochozoa is an important and seemingly difficult problem in the study of animal evolution. It may be possible to resolve these questions using multiple nuclear encoded genes. To this end, and to gather data for many additional analyses, we recommend large-scale genome sequencing be performed on members of this clade that until now has been overlooked by the genome-sequencing community.

Acknowledgments

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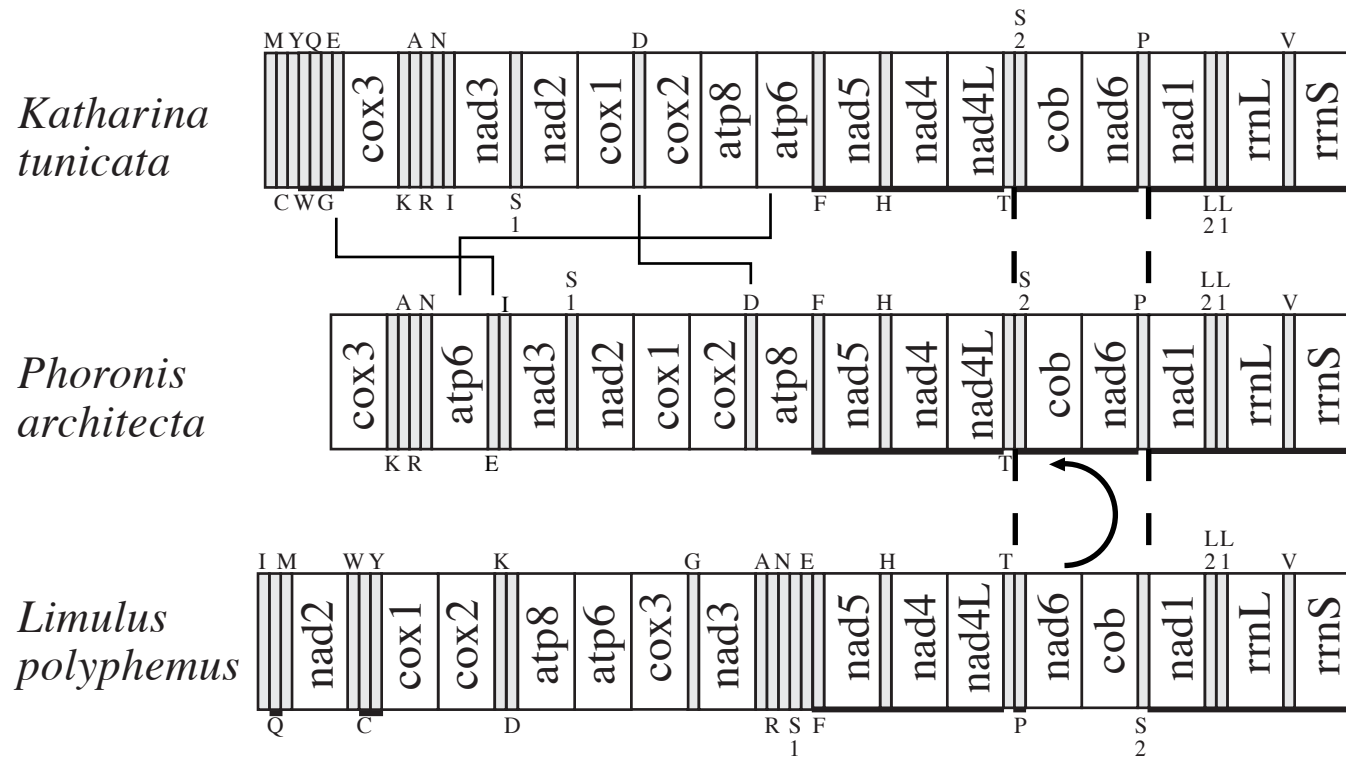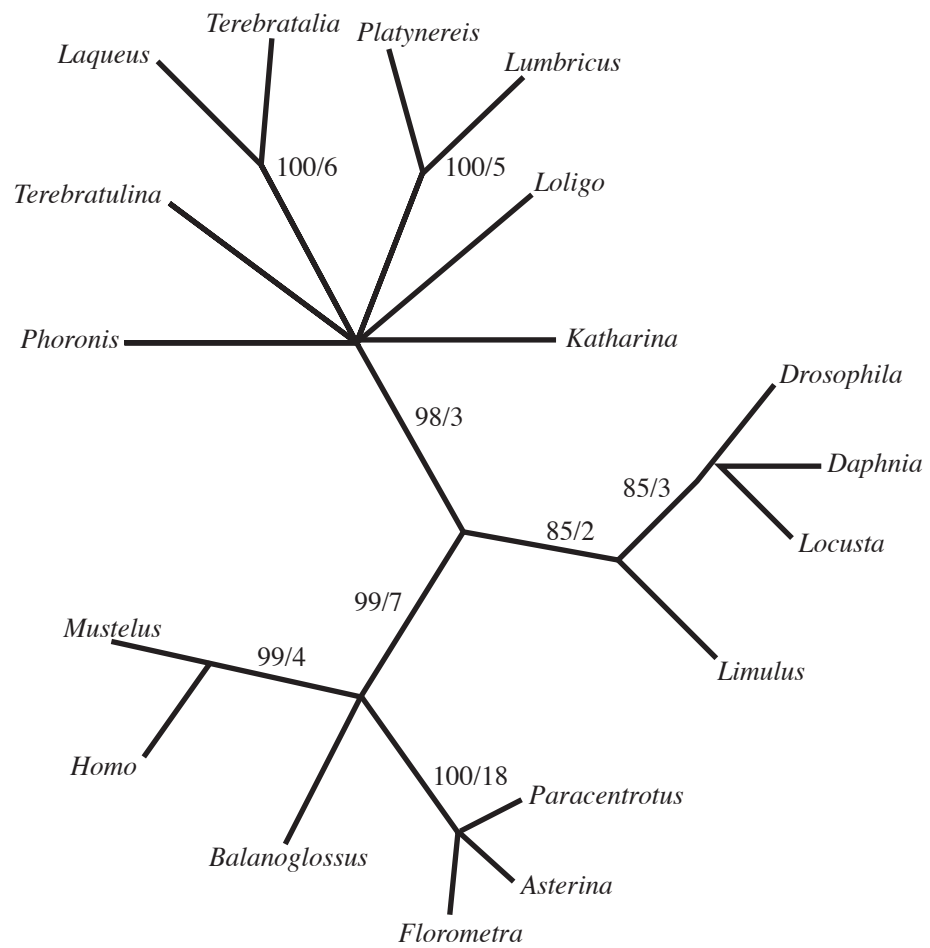
Figure legends

FɪG 1.—Comparison of the mitochondrial gene arrangement of the mollusk *Katharina tunicata* with the sequenced region of the *Phoronis architecta* mtDNA and the mtDNA of the arthropod *Limulus polyphemus*. The circular genomes have been graphically linearized at the 5' end of the *rrnS* gene. Genes encoded in a right to left transcriptional orientation are indicated by thick lines below the gene boxes. Gene rearrangements between the phoronid and chiton are indicated by thin lines. The curved arrow indicates a region of DNA—delineated by dashed lines—that is inverted in the lophotrochozoans relative to the arthropod *L. polyphemus*. One-letter amino acid abbreviations are used to label the corresponding tRNAs, excepting L1, L2, S1, and S2; these designations refer to those tRNAs with anticodons uag, uaa, ucu, and uga, respectively.
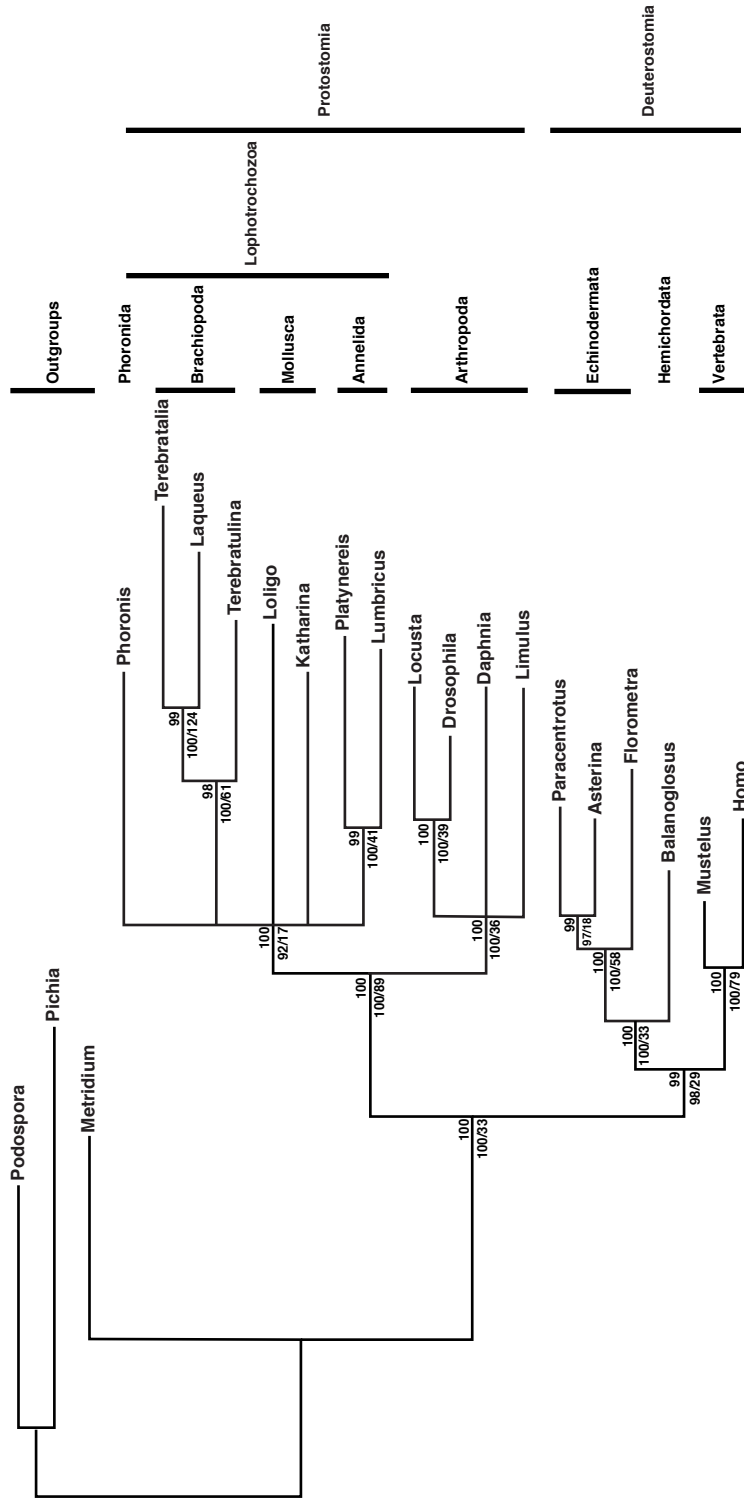
FɪG 2.—Results of parsimony analysis of the gene adjacency matrix. Branch lengths have no significance. The slash (/) separates bootstrap values on the left from Bremer support values on the right. (The latter indicates the number of evolutionary changes in the shortest tree that does not contain the node in question.) Branches with less than 80% bootstrap have been collapsed. The taxa are as follows: *Phoronis architecta* (Phoronida; phoronid), *Terebratulina retusa* (Brachiopoda; articulate brachiopod), *Laqueus rubellus* (Brachiopoda; articulate brachiopod), *Terebratalia transversa* (Brachiopoda; articulate brachiopod), *Platynereis dumerilii* (Annelida; polychaete), *Lumbricus terrestris* (Annelida; earthworm), *Loligo bleekeri* (Mollusca; squid), *Katharina tunicata* (Mollusca; chiton), *Drosophila yakuba* (Arthropoda; fly), *Daphnia pulex* (Arthropoda; water flea), *Locusta migratoria* (Arthropoda; locust), *Limulus polyphemus* (Arthropoda; horseshoe crab), *Paracentrotus lividus* (Echinodermata; sea urchin), *Asterina pectinifera*

(Echinodermata; sea star), *Florometra serratissima* (Echinodermata; feather star), *Balanoglossus carnosus* (Hemichordata; acorn worm), *Mustelus manazo* (Chordata; shark), *Homo sapiens* (Chordata; human).

FIG **3.**—Phylogenetic analyses of 2960 (including 1921 parsimony informative) aligned positions of inferred amino acids from nine of the 13 protein encoding mitochondrial genes. Numbers below branches are percent bootstrap followed by Bremer support values. Numbers above the branches are the percentage of quartet puzzling replicates supporting the node. Nodes with a bootstrap value of less than 80% (all of which have a Bremer support in the range 1-4) have been collapsed. The branch lengths are proportional to the number of substitutions calculated in the maximum-likelihood analysis. The triploblast taxa are the same as figure 2. The outgroup taxa are as follows: *Metridium senile* (cnidarian), *Podospora anserina* (fungus), and *Pichia canadensis* (fungus).

Helfenbein_fig.1

Terebratalia *Platynereis*

Laqueus *Lumbricus*

100/6 100/5

Terebratulina *Loligo*

Phoronis *Katharina*

98/3

Drosophila

Daphnia

85/3

85/2

Locusta

99/7

Mustelus

99/4

Limulus

Homo

100/18

Paracentrotus

Balanoglossus

Asterina

Florometra

Helfenbein_fig.2

Outgroups

Phoronida
Brachiopoda

Mollusca
Annelida

Arthropoda

Echinodermata
Hemichordata
Vertebrata

Protostomia

Lophotrochozoa

Deuterostomia

Podospora
Pichia
Metridium
Phoronis
Terebratalia
Laqueus
Terebratulina
Loligo
Katharina
Platynereis
Lumbricus
Locusta
Drosophila
Daphnia
Limulus
Paracentrotus
Asterina
Florometra
Balanoglosus
Mustelus
Homo

99
100/124
98
100/61
100
92/17
99
100/41
100
100/39
100
100/36
99
97/18
100
100/58
100
100/33
100
100/79
99
98/29
100
100/89
100
100/33

.10

Helfenbein__fig.3